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Altering the Length of the Lipopolysaccharide O Antigen Has an Impact on the Interaction of *Salmonella enterica* Serovar Typhimurium with Macrophages and Complement

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A panel of isogenic *Salmonella enterica* serovar Typhimurium strains that vary only in the length of the O antigen was constructed through complementation of a *wzz* double mutant (displaying unregulated O-antigen length) with one of two homologous (wzz_{ST} and wzz_{fepE}) or three heterologous (wzz_{O139} of *Vibrio cholerae* and wzz_{SF} and wzz_{pHS-2} of *Shigella flexneri*) *wzz* genes. Each gene was functional in the *S. enterica* serovar Typhimurium host and specified production of O-antigen polymers with lengths typical of those synthesized by the donor bacteria (ranging from 2 to >100 O-antigen repeat units). By use of this panel of strains, it was found that O-antigen length influences invasion/uptake by macrophage cells; this is the first time this has been shown with *Salmonella*. O-antigen length was confirmed to be related to complement resistance, with a minimum protective length of >4 and <15 repeat units. O antigen of 16 to 35 repeat units was found to activate complement more efficiently than other lengths, but this was unrelated to complement resistance. No evidence was found to suggest that modifying the length of the O-antigen polymer affected expression of the O1, O4, or O5 antigenic factors.

The lipopolysaccharide (LPS) of smooth gram-negative bacteria comprises three components—lipid A, the core oligosaccharide, and O antigen (Oag). Oag is a polymer of a sugar repeat unit (RU); the numbers of RUs attached to lipid A-core are clustered around a modal value determined by Wzz proteins (3, 26). Each *wzz* gene specifies synthesis of an Oag of a characteristic length. *Salmonella enterica* serovar Typhimurium possesses two Wzz proteins: Wzz_{ST}, which confers a modal range of 16 to 35 RUs, and Wzz_{fepE}, which confers a very long modal length estimated to be >100 RUs (20). Mutation of both of the corresponding genes resulted in a smooth, unregulated Oag phenotype characterized by predominantly short-length Oag chains and significantly compromised virulence and complement resistance (20, 21).

The identification of *wzz* genes in many pathogenic bacterial species (18) suggests that the control of Oag length confers distinct advantages. Two functional *wzz* genes have been identified in *Shigella flexneri* (11), with the product of each *wzz* gene fulfilling a distinct role in pathogenesis (11, 17, 29). The interest in the role of *S. enterica* serovar Typhimurium Oag length is further increased by recent findings that this property is under regulatory control in vivo (2, 15, 21).

Several approaches have been used to investigate the importance of *Salmonella* Oag length for pathogenic potential, but each has had its limitations. This study describes a new approach through complementation of a *Salmonella enterica* serovar Typhimurium C5 double *wzz* mutant with heterologous *wzz* genes. The resulting panel of strains was used to examine new and revisit previously investigated relationships between Oag length and virulence. Our results indicate that Oag modal length impacts upon *S. enterica* serovar Typhimurium virulence-related properties.

Construction of a panel of *S. enterica* serovar Typhimurium variants with different Oag modal lengths. C5Δwzz_{ST}Δwzz_{fepE} lacks the normal regulation of Oag modal length and produces predominantly short LPS Oag chains (Fig. 1, lanes 1 and 2) (20). To construct an isogenic panel of Oag modal-length variants, this double mutant was separately complemented with one of five *wzz* genes (wzz_{O139}, wzz_{SF}, wzz_{ST}, wzz_{pHS-2}, and wzz_{fepE}) subcloned onto the low-copy-number vector pWSK29 (32). These *wzz* genes were chosen since they confer a variety of Oag modal lengths in their normal hosts (Table 1). The proteins encoded by the selected genes showed widely varying levels of similarity (from <20% to ~70% identity).

LPS was prepared from each of the resulting complemented strains, separated in a sodium dodecyl sulfate-15% polyacrylamide gel, and detected by silver staining (20). Each of the heterologous Wzz proteins was functional in the *S. enterica* serovar Typhimurium C5 background, providing Oag modal-length regulation (Fig. 1, lanes 5 to 9) despite widely varying levels of similarity to the endogenous Wzz proteins. Each *wzz* conferred a modal length similar to that imposed in its natural host (summarized in Table 1). This demonstrates that regulation of LPS Oag modal length is a property intrinsic to Wzz proteins, confirming a previous report pertaining to *S. flexneri* (17). As judged by the level of staining, each strain had comparatively similar levels of core substitution with Oag.

The panel of Oag variants generated in this study constitutes a unique resource for investigations into the role of Oag length in *Salmonella* virulence and possesses numerous advantages

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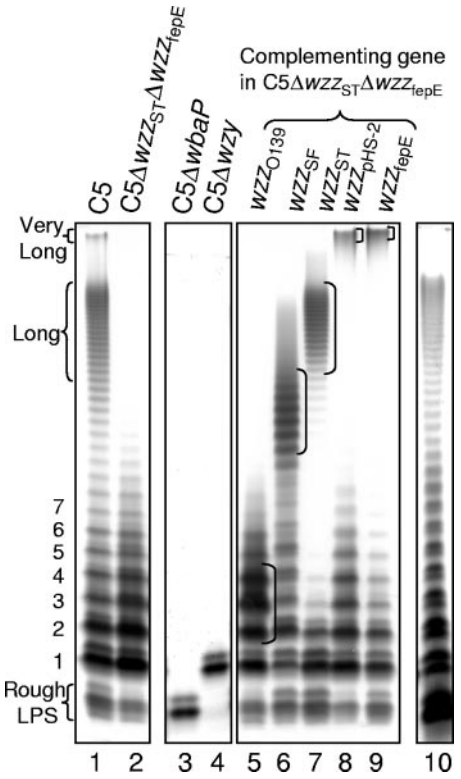

FIG. 1. Analysis of LPS from *S. enterica* serovar Typhimurium C5 Oag modal-length variants and the *wbaP* (rough) mutant. Each lane corresponds to LPS from 2×10^8 bacteria, except lanes 3 and 4 (2×10^7 bacteria). The number of Oag RUs in each band is indicated on the left of the figure. A bracket denotes the zone of modal regulation for each strain. Lane 10 corresponds to C5Δ*wbaP* (pGEM-T Easy-*wbaP*).

over other approaches for studying this phenomenon. For example, past studies have utilized strains with various mutations that confer a rough phenotype with different levels of core truncation (e.g., references 22, 27, and 35); this overlooks the fact that rough strains are avirulent. Other studies have manipulated Oag synthesis by use of sugar synthesis mutants incubated under defined growth conditions (e.g., reference 8). The set of strains described herein has the advantage of possessing stable Oag phenotypes that are not dependent on in vitro culture conditions. Past studies have also utilized purified, size-fractionated LPS for various assays (e.g., references 4, 7, and 30), but this overlooks the potential for interplay between Oag length and other outer membrane factors, as demonstrated for *S. flexneri* (17).

To serve as an additional control strain for invasion experiments, a rough mutant was constructed by mutation of the *wbaP* (*rfbP*) gene, which encodes a galactosyltransferase essential for Oag monomer synthesis (31), by use of a PCR-based mutagenesis system (6). Analysis of C5Δ*wbaP* LPS by silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Fig. 1, lane 3) showed that the strain contained lipid A-core but no Oag repeats, in contrast to C5 (lane 1) and the semirough mutant C5Δ*wzy* (lane 4) (5). Complementation with pGEM-T Easy-*wbaP* restored the smooth LPS phenotype (lane 10).

Effect of Oag length on uptake of *S. enterica* serovar Typhimurium by RAW264.7 macrophage cells. The relative uptake of strains with various Oag modal lengths was determined by competition experiments. Mixed bacterial suspensions were prepared by mixing each mutant with C5 at ~1:1 (in Dulbecco's modified Eagle's medium + 10% [vol/vol] heat-inactivated fetal calf serum) prior to addition to monolayers of RAW264.7 cells. Invasion assay conditions, including bacterial culture conditions, were as described previously (16) with modifications; contact between macrophages and bacteria was initiated by centrifugation (5 min; $650 \times g$), and after a 30-min uptake, a solution of 80 μg/ml gentamicin was added to kill extracellular bacteria (90-min incubation). Internalized bacteria were enumerated by plating detergent-lysed macrophages onto nutrient agar with appropriate antibiotics. The competitive index for each mutant was determined by dividing the output quotient (CFU mutant/CFU C5) by the corresponding input quotient. Bacteria were assayed in triplicate wells in three assays. Statistical comparisons used Student's *t* test (two tailed, two sample, equal variance). It should be noted that the culture conditions used (low oxygen tension, high osmolarity) upregulate multiple *Salmonella* SPI1 type III secretion system (TTSS)-associated genes, including those encoding structural proteins and secreted effectors (1, 16).

The absolute level of uptake for C5 varied from 4 to 16% of the bacteria added to the wells. There was a trend toward decreased bacterial ingestion as the length of Oag increased (Fig. 2), with the rough mutant taken up most efficiently ($P < 0.05$) compared to other strains. Variants with Oag lengths of 2 to 35 RUs had uptake approximately the same as or better than C5 (competitive index, ≥1), while the two strains with >90 RUs in their Oag exhibited reduced uptake (competitive index, <1).

While it has previously been shown that rough *Salmonella* strains are more readily taken up by phagocytic cells (34), this is the first time that the length of LPS Oag has been shown to

TABLE 1. Wzz genes selected for complementation of C5Δwzz_{ST}Δwzz_{fepE}

Protein	Bacterial source	Length (aa)[a]	% Identity to[b]:		Modal range in normal host species (RUs)	Estimated modal range in C5Δwzz_{ST}Δwzz_{fepE} (RUs)	Accession no.; reference
			Wzz_{ST}	Wzz_{fepE}			
Wzz_{O139}	*Vibrio cholerae* O139	335	16.4	18.2	1	2–4	X90547; 17
Wzz_{SF}	*Shigella flexneri*	325	72.0	25.8	11–17	10–15	X71970; 19
Wzz_{ST}	*S. enterica* serovar Typhimurium	327		26.6	16–35	16–35	Z17278; 20
Wzz_{pHS-2}	*S. flexneri*	368	20.7	56.5	90–100	90–100	28
Wzz_{fepE}	*S. enterica* serovar Typhimurium	378	23.0		>100	>100	NC_003197; 20

[a] aa, amino acid.
[b] Alignments were performed using ClustalW 1.8.

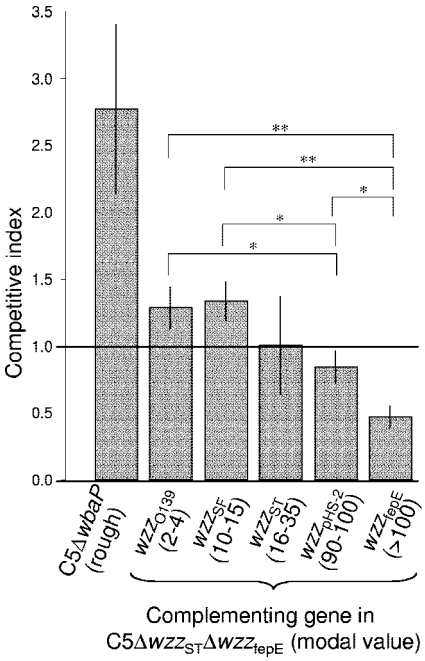

FIG. 2. Competition invasion/uptake assays using RAW264.7 macrophages. Mixtures of bacteria with different Oag modal lengths and C5 (input ratio of 1:1) were used in invasion/uptake assays, and the competitive index was subsequently calculated (input ratio/output ratio). The horizontal line (competitive index of 1) indicates the strains that have a level of uptake equivalent to that of C5 under the conditions tested. Values below this line indicate strains with reduced uptake. Histograms show means ± standard errors from three assays (statistical analysis between histograms is indicated by horizontal lines; *, $P < 0.05$; **, $P < 0.01$; Student's t test).

have an effect on invasion/uptake of *Salmonella*. This may be a result of enhanced phagocytosis through exposure of outer membrane ligands for phagocytic cell receptors, as demonstrated in the phagocytosis of rough *Escherichia coli* by dendritic cells (13). Alternatively, the longer Oag chains may inhibit invasion by masking of the SPI-1 TTSS, as recently demonstrated with *S. flexneri* (33). If each *S. enterica* serovar Typhimurium O unit contributes 1 nm to Oag length (12), a polymer of >90 repeat units may limit the exposure of the 80-nm-long TTSS needle (14). In contrast, a polymer of <36 repeat units would not inhibit this process. Other studies indicated that the length of the Oag polymer did not alter intracellular survival (data not shown).

The relative impact of the two endogenous *wzz* genes of *S. enterica* serovar Typhimurium (wzz_{ST} and wzz_{fepE}) on uptake by macrophages was also assessed by mixing strains expressing long (16- to 35-repeat-unit) and very long (>100-repeat-unit) modal lengths. To allow counterselection of strains, C5Δwzz_{ST} was complemented with pWSK29-wzz_{fepE} (very long Oag chain, tetracycline resistant), and C5Δwzz_{fepE} was complemented with pWSK29-wzz_{ST} (long Oag chain, kanamycin resistant). The LPS of each of these strains was confirmed to have a profile the same as that of the equivalent strain shown in Fig. 1 (data not shown). On average, the strain with a long Oag modal length exhibited 75% more uptake than the strain with a very long Oag modal length (a competitive index of 1.75 with a standard deviation of 0.08), confirming that the very long Oag chains inhibit uptake by macrophages.

Relationship between Oag modal length and complement resistance in *S. enterica* serovar Typhimurium. Wzz regulation of *S. enterica* serovar Typhimurium Oag length is required for complement resistance (20, 21). To determine the impact of varying the LPS Oag modal length on this phenotype, the panel of strains was incubated with 10% guinea pig serum (GPS), and viable counts were determined at 30-min intervals as described previously (21) (Table 2). The complemented strains with Oag modal lengths of >10 RUs were resistant to the activity of complement (>89% survival at 120 min). In contrast, the variant producing LPS with a range of 2 to 4 RUs was highly susceptible (8.3% survival; $P < 0.01$). This modal length provided a low level of protection compared to that provided by the double *wzz* mutant, which exhibited 2.9% survival ($P < 0.05$). These results suggest that the minimum Oag length required for complement resistance in *S. enterica* serovar Typhimurium C5 under these conditions is >4 and <15 RUs. This result is consistent with the finding that an Oag with 4 to 6 RUs was required for complement resistance in a mutant strain that was conditionally rough by virtue of an inability to synthesize mannose (8).

Relationship between Oag modal length and complement activation by *S. enterica* serovar Typhimurium. The complement-activating potentials of each of the *wzz*-complemented strains were compared by estimating the depletion of complement from GPS by inactivated bacteria. Glutaraldehyde-fixed bacteria (from log-phase cultures) were diluted to 2×10^9 CFU/ml in Veronal-buffered saline (VBS; 150 mM NaCl, 0.1% [wt/vol] sodium barbital, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH 7.35) containing 2% (vol/vol) GPS and incubated (1 h for

TABLE 2. Complement activation and serum survival of the panel of strains

Strain or complementing gene	Estimated modal length (RU) or phenotype	Residual complement activity (SE)[a]	% Survival in 10% guinea pig serum (SD)[b]
C5	16–35, >100	100	165 (40)
C5Δwzz_{ST}Δwzz_{fepE}	Smooth unregulated	ND	2.9 (3.1)
wzz_{O139}	2–4	118 (2.3)	8.3 (0.6)
wzz_{SF}	10–15	92.3 (7.1)	148 (29)
wzz_{ST}	16–35	33.2 (0.7)	165 (35)
wzz_{pHS-2}	90–100	122 (9.0)	89 (11)
wzz_{fepE}	>100	108 (5.5)	134 (26)

[a] Residual complement activity (the opposite of complement activation) was calculated by determining the depletion of complement activity from serum by each strain, expressed as a percentage of the value obtained for C5; ND, not done.
[b] Survival after 2 h of incubation (% initial numbers).

37°C). After centrifugation, the supernatants were transferred to the wells of a microtiter tray, to which were added equal volumes of 2% antibody-sensitized sheep red blood cells. After incubation at 37°C for 1 h, the reaction was stopped by the addition of VBS plus EDTA (0.03 M), and the absorbance was read at 405 nm. Data were transformed by subtracting the negative (heat-inactivated complement) control and expressed as percentages of the positive (complement-only) control to determine the percent lysis. The experiment was repeated three times with independently grown and fixed cultures employed each time. Statistical significance was assessed by one-way analysis of variance with Dunnett's posttest (GraphPad Prism, version 3.00; GraphPad Software).

For each assay, residual complement activity was expressed as a percentage of that observed for the C5 control (which averaged 59%) (Table 2). The greatest depletion of complement occurred with the wzz_{ST}-complemented strain (residual complement activity only 33% of that seen with the C5 control); this level of activity was significantly different from those seen for all other strains ($P < 0.001$). The remaining strains showed levels of complement activation similar to that observed for C5. There was no relationship between complement activation and complement sensitivity.

The relationship between Oag length and complement activation has been analyzed previously by three studies using size-fractionated, purified LPS (4, 7, 30). In two of these studies it was determined that intermediate Oag lengths have the highest complement-activating capacity (4, 30). Our results support and extend these observations by using whole bacteria. In the third of these studies it was concluded that the Oag length does not affect complement-activating potential (7). In this study, however, overlapping ranges of Oag lengths in LPS fractions may have obscured significant differences.

The reason for the relationship between Oag length and complement activation is difficult to explain. We used Western blotting to determine that the distributions of the LPS structures O1, O4, and O5 do not vary with Oag length as some other factors do (10, 23; data not shown), and therefore variable distribution of Oag modifications is unlikely to contribute to differential activation (9). As an alternative explanation, it is possible that at a certain length the *S. enterica* serovar Typhimurium LPS assumes a conformation that is sterically favorable for complement activation (24, 25).

The optimum Oag length identified for complement activation coincides with one of the Oag modal lengths normally expressed by *S. enterica* serovar Typhimurium (conferred by wzz_{ST}). Preliminary experiments comparing the uptake of two opsonized strains (C5Δwzz_{ST}Δwzz_{fepE} complemented with wzz_{ST} or wzz_{fepE}) suggest that the differential complement activation does not translate to increased rates of uptake by RAW264.7 cells (data not shown). Further experiments are required to elucidate the significance of this relationship.

Conclusions. A panel of isogenic strains with variation in Oag modal length was developed. This panel constitutes a unique resource for investigations into the role of Oag length in *Salmonella* virulence. It was used to show that O-antigen length affects a key feature of *Salmonella* virulence (entry into cells), and it allowed an independent determination of the minimum length of Oag need to confer resistance to complement. The relationship between Oag length and complement activation was clarified, and this was shown to be unrelated to distribution of epitopes on the O-antigen chains.

It is possible to speculate on the advantage conferred to *S. enterica* serovar Typhimurium by synthesis of bimodal LPS. The very long Oag chains may be present for complement resistance or to reduce complement activation, while long Oag chains could disperse the very long chains to facilitate uptake by macrophages and other cells. Furthermore, regulation of the very long Oag chains in *S. enterica* serovar Typhimurium may impart particular virulence characteristics at different stages of infection (21). Such a relationship would be similar to the model proposed for *S. flexneri*, where very long chains provide serum resistance, while short chains disperse the very long chains to aid in the correct exposure of the virulence protein IcsA (17).

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